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RESEARCH COMMUNICATION

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Fisetin, a major component derived from mulberry (*Morus australis* Poir.) leaves, prevents vascular abnormal contraction

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Abstract

Mulberry (*Morus australis* Poir.) leaves have long been consumed in the form of tea or tincture especially in Asia, owing to their high antioxidant and blood pressure-regulating properties. Although it is thought that vascular abnormal contraction may be involved in the blood pressure-suppressing effect, the effect of mulberry on vascular abnormal contraction is still unknown. Therefore, we investigated mulberry leaves as a potential source of bioactive compounds that prevent vascular abnormal contraction. Mulberry leaves were divided into fresh leaves and tea leaves and further classified according to the age of the tree: more or less than 20 years old, into roasted and unroasted. Mulberry fruits were also evaluated. We assessed the preventive effect of mulberry extracts on vascular abnormal contraction. Extracts from mulberry leaves of trees more than 20 years old showed a strong preventive effect on vascular abnormal contraction of human coronary artery smooth muscle cells. Therefore, to identify the active components in mulberry leaves, we fractionated the active fractions by gel filtration chromatography and reversed-phase high-performance liquid chromatography. The active fraction was further analyzed by mass spectrometry and nuclear magnetic resonance; an active component of the mulberry leaf extract was fisetin. In addition, our results indicated that the hydroxyl group at the C-3 position of fisetin is crucial for its activity. These results prove that fisetin is effective in preventing vascular abnormal contraction. Overall, mulberry leaves and fisetin are expected to be used in a wide range of fields such as functional foods, nutraceuticals, and drug targets.

KEYWORDS

fisetin, mulberry leaf, nutraceutical, preventative medicine, vascular abnormal contraction

Abbreviations: Ctrl, control; DMSO, dimethyl sulfoxide; HCASMCs, human coronary artery smooth muscle cells; HMBC, heteronuclear multiple bond correlation; HPLC, high-performance liquid chromatography; HSQC, heteronuclear single quantum correlation; LC-ESI-MS/MS, liquid chromatography–electrospray ionization-tandem mass spectrometry; ND, not detected; NMR, nuclear magnetic resonance; PAECs, porcine aortic endothelial cells; RT, retention time; SD, standard deviation; SPC, sphingosylphosphorylcholine; TFA, trifluoroacetic acid.

[Correction added after first online publication on November 6, 2021. Funding information section has been modified.]

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1 | INTRODUCTION

Vascular diseases, such as cardiovascular and cerebrovascular disease, are among the leading cause of death globally.[1,2] These diseases are caused by abnormalities in the blood vessels in the heart, brain, and other organs, resulting in an impaired blood flow. The causes of vascular diseases can be broadly classified into three categories: arteriosclerosis, abnormal contraction, and hemorrhage/inflammation. Recently, the incidence of vascular diseases caused by abnormal contraction has been increasing.[3] Blood vessels are composed of three layers: innermost intima, media, and outermost adventitia, and abnormal contraction occurs in the vascular smooth muscle cells that make up the media. Normal contraction is Ca^{2+}-dependent and maintains blood pressure and flow by repeating the cycle of contraction and relaxation, whereas abnormal contraction is Ca^{2+} -independent and causes localized irreversible contraction.[4] Vascular abnormal contraction is a well-known cause of sudden death in healthy people of all ages.[5] Although some mechanisms underlying the abnormal contractions have been clarified,[6] the detailed molecular mechanisms are still unknown, making the development of therapeutic agents difficult. Consequently, there are no effective components to inhibit abnormal contractions, and new preventive methods and drug targets should be developed and identified, respectively.

The preferred approach to prevent vascular abnormal contraction is the intake of functional foods, nutraceuticals, and/or preventative medicines on a daily basis. Recently, bioactive compounds contained in common foods and beverages have been attracting attention as therapeutic agents for various diseases.[7,8] For example, antioxidants in red wine have been suggested to have a functional effect on blood vessels.[9] Various other foods have been reported to regulate physiological functions, such as the vascular endothelial function-improving effects of Sakurajima daikon (*Raphanus sativus* "Sakurajima Daikon")[10,11] and the antioxidant effects of *Capsicum annuum*.[12] Mulberry (*Morus australis* Poir.) leaves are cultivated worldwide, including Asia and Europe,[13] and are used to treat sore throats[14] and inflammation.[15] They also have high antioxidant activity,[16,17] and their contents have been shown to regulate blood pressure.[18–20] Although it is thought that vascular abnormal contraction may be involved in the blood pressure-suppressing effect, the effect of mulberry leaf extracts on vascular abnormal contraction remains unclear. Therefore, we hypothesized that mulberry leaf extract can prevent vascular abnormal contraction of human coronary artery smooth muscle cells (HCASMCs). This study was conducted to clarify the preventive effects and identify the active component of mulberry leaf extracts.

2 | EXPERIMENTAL PROCEDURES

2.1 | Materials

Mulberry samples were kindly provided by Miyazono Tea Company (Kagoshima, Japan). HCASMCs, HuMedia-SB2, and HuMedia-SG2 media for culturing vascular smooth muscle cells, and HuMedia-EB2 and HuMedia-EG2 media for culturing vascular endothelial cells were purchased from Kurabo Co., Ltd. (Osaka, Japan). Porcine aortic endothelial cells (PAECs) were purchased from Cosmo Bio Co., Ltd. (Tokyo, Japan). Sphingosylphosphorylcholine (SPC) and Fluo3-AM were purchased from Sigma-Aldrich Co. LLC. (St. Louis, MO, USA). Dimethyl sulfoxide (DMSO)-d_6 (0.03% tetramethylsilane) used in nuclear magnetic resonance (NMR) analysis was purchased from ACROS ORGANICS (Antwerp, Belgium), and fisetin was purchased from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan).

2.2 | Preparation of mulberry leaf and fruit extracts

Mulberry leaves were obtained from trees of various ages. To consider the effect of tree age, fresh mulberry leaves were divided into two groups based on the age of the tree from which they were collected: more or less than 20 years old. Mulberry tea leaves, which were prepared by steaming mulberry leaves for 30 s and drying them in hot air at 80°C, were divided into two groups: roasted and unroasted. Mulberry fruits were also freeze-dried, powdered, and evaluated for their functional effects. Samples were extracted with 70% ethanol, and the concentration was adjusted to 30 mg/ml in ethanol. After stirring, the mixture was sonicated for 10 min, centrifuged at $698 \times g$ for 10 min at 4°C (Kubota Shoji Co., Ltd., Tokyo, Japan), and the supernatant was collected. Ethanol (70%) was added to the pellet, and the extraction procedure was repeated twice. The resulting extract was dried in a vacuum centrifuge (IWAKI Co., Ltd., Tokyo, Japan) and stored at −20°C until use.

2.3 | Cell culture

HCASMCs were cultured in HuMedia-SB2 supplemented with 5% fetal bovine serum, 0.5 ng/ml human epidermal growth factor, 2 ng/ml human fibroblast growth factor-B, 5 μg/ml insulin, 50 μg/ml gentamycin, and 50 ng/ml amphotericin B (HuMedia-SG2). PAECs were cultured in Humedia-EB2 supplemented with 2% fetal bovine serum, 10 ng/ml human epidermal growth factor, 1.34 μg/ml

hydrocortisone hemisuccinate, 5 ng/ml human fibroblast growth factor-B, 10 μg/ml heparin, 50 μg/ml gentamycin, and 50 ng/ml amphotericin B (HuMedia-EG2). Cells were cultured in an incubator (PHC Holdings Co., Ltd., Tokyo, Japan) at 37°C under 5% CO_2. HCASMCs within eight passages were utilized in the experiments.

2.4 | Effect of mulberry extracts on abnormal contraction of vascular smooth muscle cells

HCASMCs (2.0×10^4 cells/well) were seeded into 24-well plates and incubated at 37°C under 5% CO_2 until they reached 80%–90% confluency; mulberry samples were added at 24 h after cell seeding. Once confluency was reached, the supernatant was removed, and 100 μl/well of HuMedia-SB2 with mulberry samples was added into each well. The cell cytoplasm was stained by adding 100 μl/well of 6 μmol/L Fluo3-AM (final concentration: 3 μmol/L), and incubated for 1 h at 37°C. Next, 2 mmol/L $CaCl_2$ (200 μl/well, final concentration: 1 mmol/L) was added, and the cells were incubated for another 30 min at 37°C. Following incubation, the supernatant was removed, and the mulberry sample was added again to each well. To measure the short-term effect of the mulberry extract, the cells were incubated with the extract for 30 min before inducing abnormal contraction by adding SPC. Based on previous studies, the final SPC concentration used was either 30 or 100 μmol/L.[21,22] Abnormal contractions were evaluated by taking images of the cells using an inverted microscope CKX53 (Olympus Corporation, Tokyo, Japan) for fluorescence imaging before and after adding SPC. To compare the degree of occurrence of abnormal contraction, the cell surface area of the image was calculated by measuring the amount of Fluo3-AM using ImageJ ver1.52 software (NIH, Bethesda, MD, USA),[23–25] and the relative change in the cell surface area before and after abnormal contraction was calculated.

2.5 | Fractionation and purification of mulberry extracts

The concentrated and dried mulberry extracts prepared as described in Section 2.2 were dissolved in 2.5 ml ultrapure water, stirred, and sonicated for 15 min to prepare the sample. The prepared sample was passed through a gel filtration column PD-10 (Cytiva, Tokyo, Japan), and the eluted fraction was collected as Fraction 1. Subsequently, 4×2.5 ml of ultrapure water was added to each sample, passed through the column, and Fractions 2–5 were collected. The remaining sample was eluted with 2.5 ml of 1 M NaCl, and the final eluted fraction was collected as Fraction 6 (including the remaining solution). The obtained fractions were concentrated and dried using a freeze dryer (Tokyo Rika Instruments Co., Ltd., Tokyo, Japan). These fractions were evaluated as described in Section 2.4. Fractions with a high activity were dissolved in equal volumes of 0.1% trifluoroacetic acid (TFA) and acetonitrile, passed through a 0.45-μm filter (Advantech Toyo Co., Ltd., Tokyo, Japan), and fractionated by high-performance liquid chromatography (HPLC, LC-2000Plus series; JASCO Corporation, Tokyo, Japan). Fractions were collected using a C18 reversed-phase column (TSKgel ODS-100Z, 5 μm, 4.6 mm ID, 15 cm; Tosoh Corporation, Tokyo, Japan), with the column oven set to 40°C, UV detector set to an absorbance of 320 nm, and flow rate of 1.0 ml/min. The mobile phase was 0.1% TFA in distilled water (A) and 100% acetonitrile (B). The HPLC gradient was run at 10% B from 0 to 10 min, followed by an increase to 100% B from 10 to 20 min, and held at 100% B from 10 to 20 min. After collecting the eluent at 0–10 min (Fraction X), 10–20 min (Fraction Y), and 20–30 min (Fraction Z), the fractions were concentrated and dried using a rotary evaporator (Tokyo Rika Instruments Co., Ltd.) and a vacuum centrifuge. Each fraction obtained by HPLC fractionation was evaluated as described in Section 2.4.

2.6 | Identification of active components

The high-activity fraction obtained by HPLC fractionation described in Section 2.5 was dissolved in an equal volume of mobile phase and passed through a 0.45-μm filter, after which compound identification was performed using an LC20 HPLC system (Shimadzu Corporation, Kyoto, Japan) and a QTRAP® liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS) 3200 system (1.7 with HotFix 2, AB Sciex Pte. Ltd., Framingham, MA, USA). The analytical column was a C18 reversed-phase column (TSKgel ODS-100Z, 5 μm, 4.6 mm ID, 15 cm; Tosoh Corporation) equipped with a UV detector at a flow rate of 0.4 ml/min and with an injection volume of 10 μl. Mobile phases were distilled water (A) and acetonitrile (B), and the HPLC gradient condition was 50% B for 10 min. MS/MS detection was performed in positive and negative ion modes. Finally, detection was performed in the positive ion mode. Other measurement conditions were as follows: scan type, EPI; curtain gas, 20; ion spray voltage, 5500; temperature, 500°C; ion source gas 1, 40; ion source gas 2, 50; and collision energy, 35. The resulting fragment ions were matched to identified compounds using the METLIN and MassBank databases.

The chemical structures of the identified compounds were verified by ^{1}H NMR, ^{13}C NMR, and 2D NMR with heteronuclear single quantum correlation (HSQC) and heteronuclear multiple bond correlation (HMBC) using an NMR 700 MHz instrument (Bruker Corporation, Billerica, MA, USA).[26] The measured samples were dissolved in DMSO-d_6. Active components were quantified using EXTREMA (Japan Spectroscopy Co., Ltd., Tokyo, Japan). The compounds were eluted from a C18 reversed-phase column (TSKgel ODS-100Z, 5 μm, 4.6 mm I.D., 25 cm; Tosoh Corporation) maintained at 40°C with a UV detector set to acquire data at 320 nm, a flow rate of 1.0 ml/min, and an injection volume of 10 μl. The mobile phases were 0.1% TFA in distilled water (A) and 100% methanol (B). The HPLC gradient conditions were 0–12 min at 50% B, followed by 12–17 min at 100% B.

2.7 | Vascular endothelial cell permeability test

The permeability of the active compound across endothelial cells was evaluated to ensure it could penetrate the vascular tissue. A 0.45-μm pore-sized transmembrane filter, Intercell TP (Kurabo Co., Ltd., Osaka, Japan) was coated with 30-fold diluted Cellmatrix Type I-A (Nitta Gelatin Co., Ltd., Osaka, Japan). The coated inner chamber of the intercell was seeded with PAECs (2.0×10^5 cells/well in 300 μl/well HuMedia-EG2) and incubated for approximately 24 h in an incubator at 37°C under 5% CO_2. At 24 h after seeding, 100 μl/well of the active compound was added and incubated for approximately 24 h. The supernatant was aspirated from the inner chamber of the intercell assay apparatus and phenol red-free HuMedia-EB2 (200 μl/well) was added. Similarly, 500 μl/well HuMedia-EB2 was added to the outer chamber of the intercell assay apparatus. To test membrane permeability, 50 μmol/L FITC-dextran (Sigma-Aldrich Co. LLC.) and the active compound were added to the inner chamber of the intercell assay apparatus (100 μl/well) and incubated at 37°C. After 1, 6, 12, 24, and 84 h of incubation, the outer chamber liquid was collected, and fluorescence was measured at 535 nm using an Infinite F NaNo+ spectrofluorometer (TECAN, Männedorf, Switzerland).[27] The collected outer chamber solution was freeze-dried and quantified by HPLC.

2.8 | Statistical analysis

Statistical analyses were performed using Microsoft Office Excel 2019. Quantitative analysis of all components was repeated four times independently. Differences between groups were assessed using a two-sided Student's *t*-test with an α level of 0.05. Data are presented as mean ± standard deviation (SD). Results with $p < 0.05$ were considered statistically significant.

3 | RESULTS

3.1 | Preventive effect of mulberry extracts on vascular abnormal contraction

We assessed the preventive effect of mulberry extracts on vascular abnormal contraction. HCASMCs and mulberry samples were incubated together for 30 min before adding SPC to induce abnormal contraction; and we observed cell morphology before and after inducing abnormal contraction. As components that prevent vascular abnormal contractions have not been identified, a positive control could not be established. Instead, we evaluated their effects compared with those of the control samples. The relative surface area of SPC-treated cells in the absence of mulberry extract (Ctrl) was 37.6% compared with that before abnormal contraction (100%). In contrast, the surface area of cells treated with extracts of mulberry leaves from trees >20 years old and unroasted tea leaves was 75.2% and 69.4%, respectively, compared with that before abnormal contraction (Figure 1A). This result suggests that these extracts prevented abnormal contractions. Next, we investigated whether this preventive effect changes in response to longer incubation with mulberry extracts (60 h, Figure 1B). The results showed that extending the preincubation time with mulberry extracts did not change the preventive effects, but the protective effect of mulberry extracts was reduced when fresh extract was not added at the time of SPC addition (Figure 1C). These results suggest that continuous exposure of HCASMCs to mulberry leaf extract prevents their abnormal contraction.

3.2 | Fractionation and purification of active components extracted from mulberry leaves

Gel filtration chromatography was performed to fractionate the active fraction from mulberry leaves of >20 years old trees. Fraction 1 was excluded because it had a high-molecular weight (over 5000 Da) and could not be absorbed orally; Fraction 2 showed cytotoxicity. Fraction 6 showed the strongest preventive effect against abnormal contraction (Figure 2A). We also examined the preventive effects of fractions 6-X, 6-Y, and 6-Z, isolated from fraction 6 by reversed-phase HPLC; fraction 6-Y showed strong effects (Figure 2B).

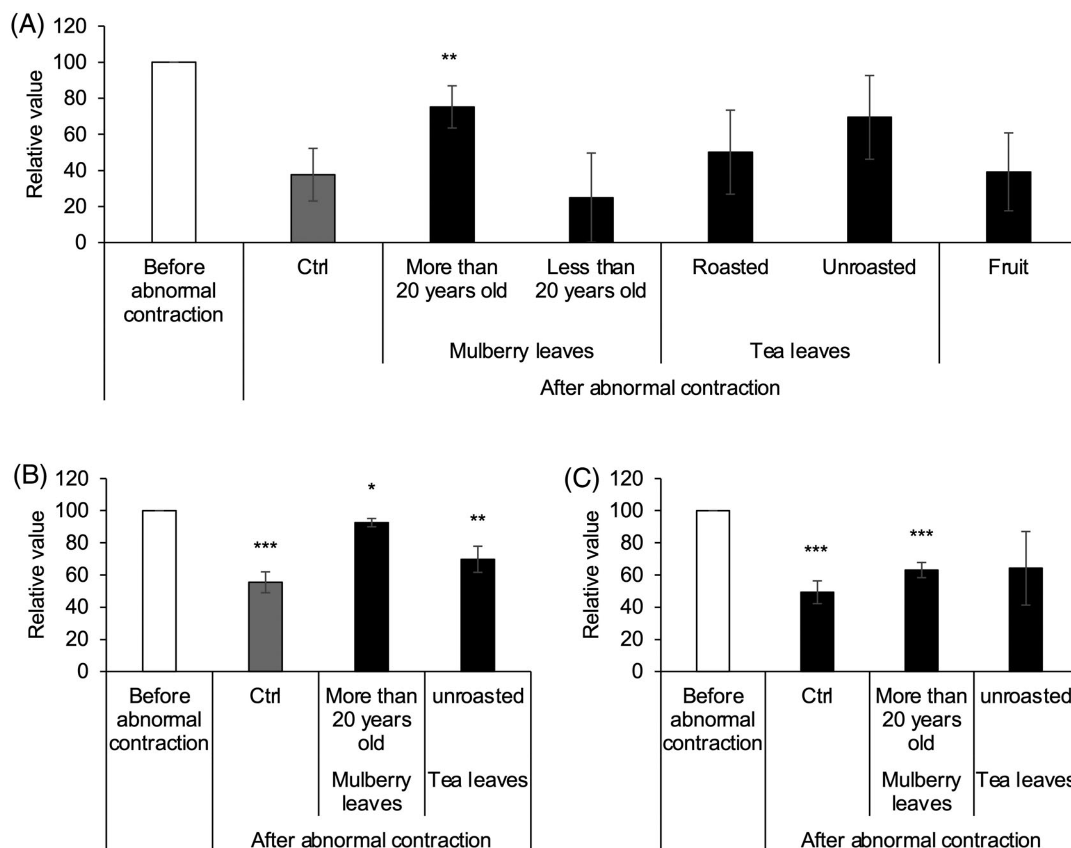

FIGURE 1 Preventive effect of mulberry leaf extracts on vascular abnormal contraction. Effects of mulberry leaf extracts on sphingosylphosphorylcholine (SPC)-induced vascular abnormal contraction after incubation for 30 min (A) and 60 h (B), and when fresh leaf extract was not added at the time of SPC-induction following incubation with the mulberry extracts (C). Data expressed as mean ± SD ($n = 4$); ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$ versus Ctrl (induction of abnormal contraction without the addition of mulberry extract)

3.3 | Identification of the active component in mulberry leaves from trees >20 years old

To identify the active component in mulberry leaf extracts conferring preventive effects against abnormal contraction, Fraction 6-Y was analyzed using LC-ESI-MS/MS. As a result, fisetin and luteolin (Figure 3A), which have the same molecular weight (m/z $[M + H^+]$ 286.8) but different hydroxyl group positions, were considered as candidate components. Fisetin has a hydroxyl group at position 3 on the C-ring of the flavonoid skeleton, whereas the hydroxyl group in luteolin is at position 5 on the A-ring (see structures in Figure 3A). Therefore, we first checked the retention time of both compounds using a purified standard; luteolin was not present in mulberry leaf extract of trees >20 years old (Figure 3B). Furthermore, we determined the chemical structure of the candidate compound by NMR. ^{1}H NMR (700 MHz, DMSO-d_6): δ 7.92 (1H, dd, J = 7.0 Hz, H-5), 6.90 (1H, m, H-6), 6.90 (1H, m, H-8), 7.69 (1H, d, J = 7.0 Hz, H-2′), 6.89 (1H, m, H-5′), and 7.54 (1H, dd, J = 7.0 Hz, H-6′); ^{13}C NMR (700 MHz, DMSO-d_6): δ 144.9 (C-2), 137.0 (C-3), 171.8 (C-4), 126.3 (C-5), 114.6 (C-6), 162.2 (C-7), 101.7 (C-8), 156.1 (C-9), 114.0 (C-10), 122.3 (C-1′), 114.8 (C-2′), 144.9 (C-3′), 147.1 (C-4′), 115.4 (C-5′), and 119.47 (C-6′). HMBC spectroscopy showed the coupling correlation of H-5 (δ_H 7.91–7.92) with C-4 (171.81), C-7 (162.20), C-8 (101.67), and C-9 (156.14); H-6 (δ_H 6.90) with C-4, C-5 (126.32), C-7, C-8, and C-9; H-8 (δ_H 6.87–6.90) with C-6 (114.55), C-7, C-9, and C-10 (114.02); H-2′ (δ_H 7.68–7.69) with C-1′ (122.33), C-3′ (144.86), C-4′ (147.10), and C-2 (144.89); H-5′ (δ_H 6.87–6.89) with C-1′, C-2′ (114.77), C-3′, C-4′, and C-6′ (119.47); H-6′ (δ_H 7.53–7.54) with C-3′, C-4′, and C-5′ (115.42). These results confirmed the long-range coupling of fisetin. The HSQC spectroscopy results showed that aromatic protons coupled with the corresponding carbons. The H-5 proton (δ_H 7.91–7.92) coupled with C-5 (δ_C 126.32), H-6 (δ_H 6.90) with C-6 (δ_C 114.55), H-8 (δ_H 6.87–6.90) with C-8 (δ_C 101.67), H-2′ (δ_H 7.68–7.69) with C-2′ (δ_C 114.77), H-5′ (δ_H 6.87–6.89) with C-5′ (δ_C 115.42), and H-6′ (δ_H 7.53–7.54) with C-6′ (δ_C 119.47). These results indicate that fisetin

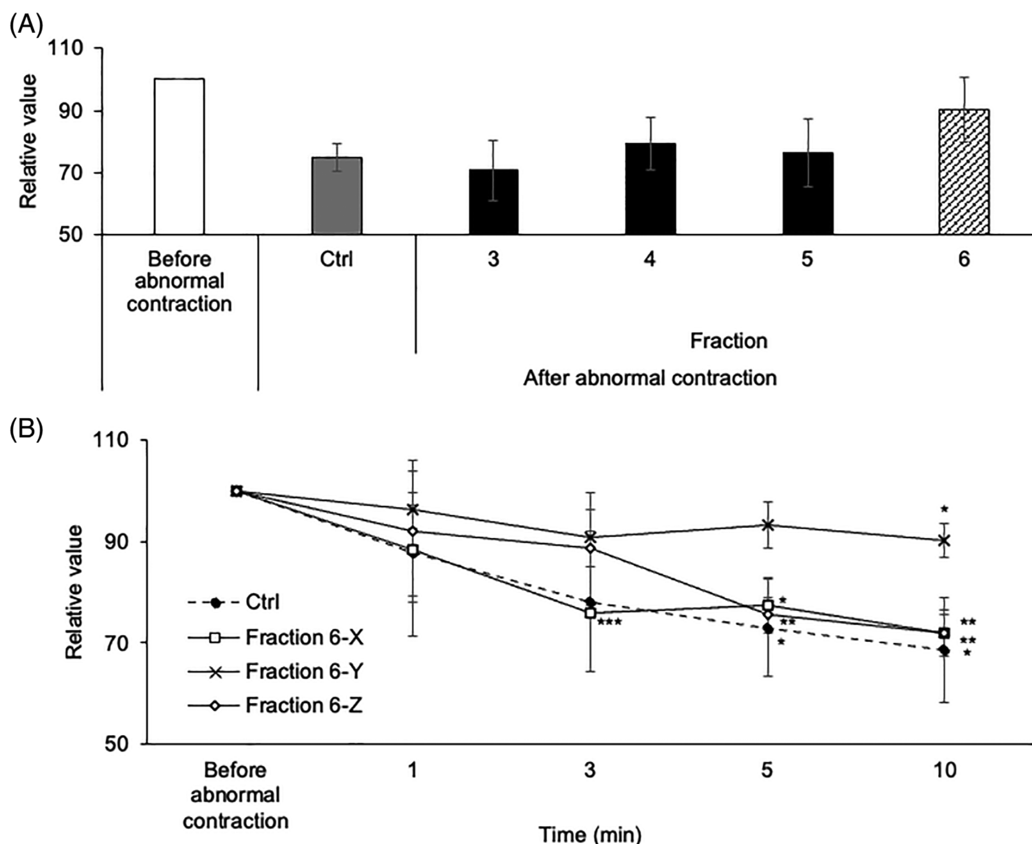

FIGURE 2 Preventive effect of mulberry leaf extract fractions on vascular abnormal contraction. (A) Effect of gel filtration chromatography Fractions 3–6 on abnormal contraction. (B) Effect of reversed-phase HPLC Fractions 6-X, 6-Y, and 6-Z on abnormal contraction. Data are expressed as mean ± SD ($n = 4$); ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$ versus Ctrl (induction of abnormal contraction without addition of mulberry extract fractions)

is an active component in mulberry leaf extract and may prevent vascular abnormal contraction.

3.4 | Determination of fisetin concentration in mulberry leaf extract from trees >20 years old and its optimal concentration

The fisetin concentration in the leaves of mulberry trees >20 years old was 283.61 μg/g dry powder (Table 1). Unexpectedly, the highest fisetin concentration was found in trees <20 years old. In addition, flavonoids are generally found in plants as glycosides; however, in mulberry leaves, fisetin was not found as a glycoside but as an aglycon (data not shown). Furthermore, mulberry leaves have a higher fisetin concentration than strawberries and apples, which are rich sources of fisetin.[28] Therefore, as the aglycon of fisetin was considered the active component of mulberry leaves, we also investigated the optimal concentration of fisetin to prevent abnormal contraction induced by SPC using fisetin preparations, and estimated it as 1 μmol/L (Figure 3C,D).

3.5 | Vascular permeability and structure–activity relationship of fisetin

Fisetin should penetrate the intima layer of vascular endothelial cells to act on vascular smooth muscle cells. Therefore, we evaluated the permeability of fisetin; 53.3% of the total fisetin permeated the endothelial cells by 1 h and 85.2% permeated by 84 h (Figure 4A). Thus, fisetin can penetrate vascular endothelial cells and act directly on vascular smooth muscle cells, although its penetration requires time. Furthermore, we evaluated the structure–activity relationship using flavonoids with similar chemical structures to identify the active site of fisetin (Figure 4B). Fisetin has a hydroxyl group at C-3 of the flavonoid skeleton, whereas luteolin has a hydroxyl group at A-5, and quercetin at C-3 and A-5. The number of hydroxyl groups is the same in the B-ring of fisetin, luteolin, and quercetin. The effect of the location of

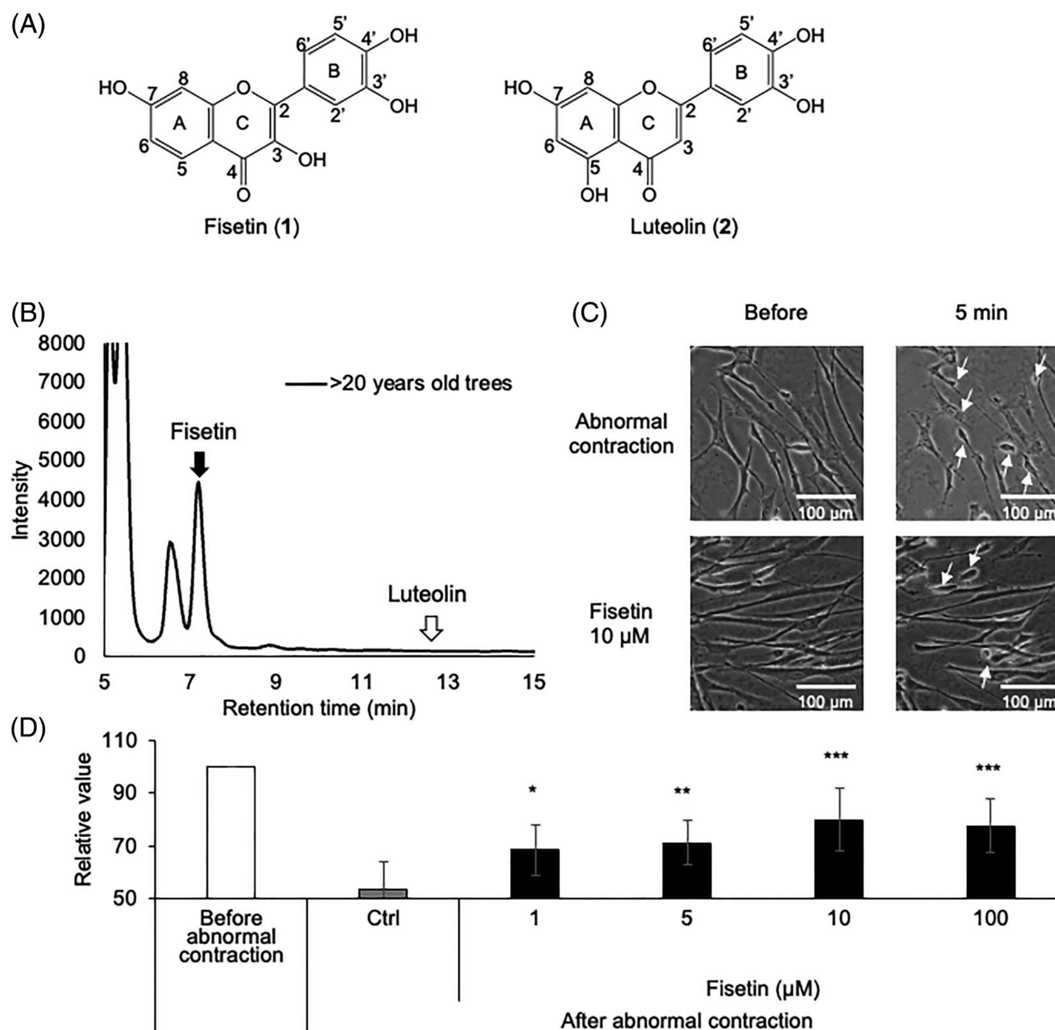

FIGURE 3 Fisetin concentration in extract prepared from mulberry leaves of more than 20 years old trees and its optimal concentration. (A) Chemical structures of fisetin (1) and luteolin (2). (B) Confirmation of fisetin and luteolin in the leaves of old mulberry trees using HPLC. The retention time (RT) for each standard was RT 7.2 for fisetin and RT 12.5 for luteolin. (C) Images of the preventive effect of fisetin on abnormal contraction of human coronary artery smooth muscle cells. (D) Preventive effect of fisetin concentration on abnormal contraction. Data expressed as mean ± SD ($n = 4$); ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$ versus Ctrl (induction of abnormal contraction without addition of fisetin)

TABLE 1 Quantitative analysis of fisetin in different parts of mulberry, strawberry, and apple samples

Sample			Fisetin (μg/g dry weight)		
			Mean	SD	*p*
Mulberry	Leaf	>20 years old	283.61	1.97	***
		<20 years old	606.21	9.26	***
	Tea leaf	Roasted	153.67	1.92	—
		Unroasted	175.81	2.26	***
	Fruit		12.49	3.70	***
Strawberry	Fruit		0.02	0.01	***
	Leaf		ND	—	—
Apple	Fruit		ND	—	—
	Peel		ND	—	—

Note:***$p < 0.001$, **$p < 0.01$, *$p < 0.05$ versus roasted tea leaves.
Abbreviations: ND, not detected; SD, standard deviation.

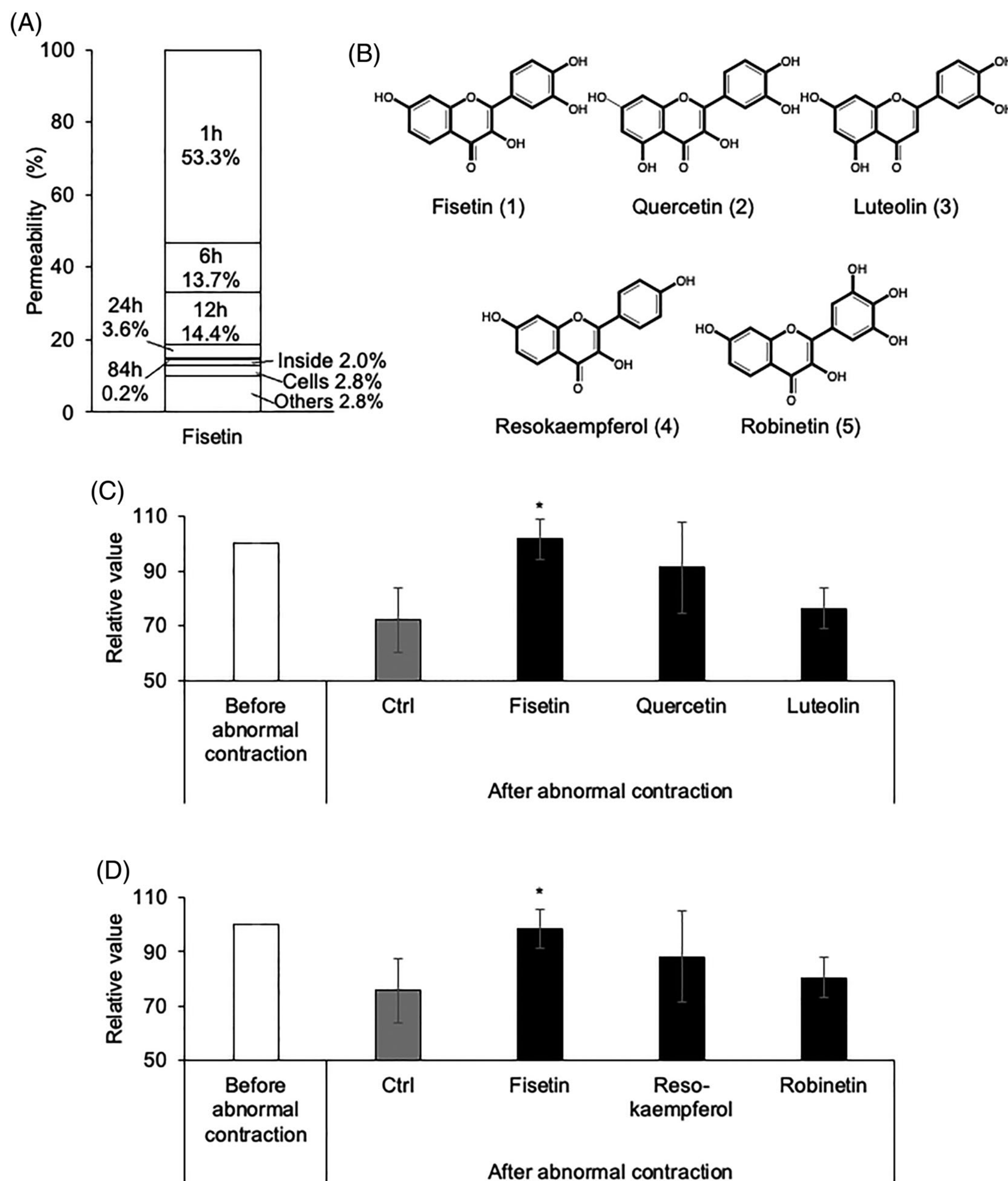

FIGURE 4 Fisetin structure–activity relationship and permeability into the vasculature. (A) Vascular endothelial cell permeability of fisetin. (B) Chemical structures of compounds compared to determine the structure–activity relationship of fisetin. (C) Preventive effects of the hydroxyl group arrangement on the A-ring and C-ring on vascular abnormal contraction. Fisetin: hydroxyl group at C-3 position, quercetin: hydroxyl group at C-3 and A-5 positions, luteolin: hydroxyl group at A-5 position. (D) Preventive effects of the number of B-ring hydroxyl groups on vascular abnormal contraction. Fisetin: 2 hydroxyl groups, resokaempferol: 1 hydroxyl group, robinetin: 3 hydroxyl groups. Data expressed as mean ± SD ($n = 4$); ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$ versus Ctrl (induction of abnormal contraction without the addition of samples)

hydroxyl groups on the A- and C-rings on abnormal contraction was evaluated using each of the three compounds as described in Section 2.4. All compounds prevented vascular abnormal contraction, with fisetin conferring the greatest protection, followed by quercetin and luteolin. The location of the hydroxyl group at C-3 appeared to be essential for the protective effect of this molecule (Figure 4C). To assess the effect of the number of hydroxyl groups in the B-ring, the protective effects of the compounds resokaempferol (1 hydroxyl group), fisetin (2 hydroxyl groups), and robinetin (3 hydroxyl groups) were also assessed. In all three compounds, the position and number of hydroxyl groups on the A- and C-rings were the same. There was no significant difference in the protective effect of resokaempferol, fisetin, and robinetin on vascular abnormal contraction (Figure 4D).

4 | DISCUSSION

We hypothesized that mulberry leaf extracts prevent vascular abnormal contractions in HCASMCs. We found that the extract of mulberry leaves from trees >20 years old prevents vascular abnormal contraction. However, the preventive effect decreased when the mulberry leaves were roasted and when mulberry leaf extract was not added at the time of contraction despite exposure to mulberry leaves for a long time (60 h). The difference in the preventive effects of roasted and unroasted tea leaves can be attributed to the effect of the roasting process; for example, it is known that iminosaccharides[29] and trigonelline[11] are degraded during the roasting process and that high-temperature drying decreases the antioxidant activity of mulberry leaves.[30] Therefore, roasting may have affected the degradation of key components of mulberry leaves. The preventive effects of the mulberry leaf extract on cells in culture did not change over time; instead, it was important that the extract was present when abnormal contraction was induced by SPC. These observations suggest that regular intake of mulberry leaves as a functional food, nutraceutical, and/or preventative medicine can prevent vascular abnormal contraction.

In addition, we investigated the active components in mulberry leaves and found that fisetin, a flavonoid, is the active component. Fisetin regulates vascular contraction by inhibiting the activity of Rho kinase in a vascular endothelial cell-independent manner,[31] although no study has isolated fisetin from mulberry leaves. In contrast, luteolin is a known constituent of mulberry leaves that can improve vascular function by inhibiting arginase, thereby improving vascular endothelial function.[32] However, to the best of our knowledge, there are no reports on the preventive effect of either compound on vascular abnormal contraction. Therefore, we investigated the effect of fisetin in preventing abnormal contractions. As fisetin needs to penetrate endothelial cells to act directly on vascular smooth muscle cells, we confirmed its endothelial cell permeability. Fisetin penetrated vascular endothelial cells, and it is thought that it can prevent vascular abnormal contraction by directly acting on vascular smooth muscle cells in vivo. However, as the timing of abnormal contraction cannot be predicted, it is necessary to regularly include mulberry leaves in the diet to prevent vascular abnormal contraction.

Furthermore, fisetin was found to be abundant in mulberry leaves. However, more fisetin was found in trees <20 years old than those >20 years old. As the expression of antioxidant enzymes in mulberry leaves changes depending on the growth stage of the tree,[33] it is possible that, although the leaves of trees <20 years old have a high concentration of fisetin, which can prevent vascular abnormal contraction, the presence of other substances offsets the protective effects. Thus, there may be a mechanism that inhibits the preventive effect of fisetin in the leaves from trees <20 years old on vascular abnormal contraction. For these reasons, if mulberry leaves are to be used as a dietary source of fisetin, it is important that the details, including the age of the parent tree and heat treatment of the leaves, are checked. In addition, we investigated the optimal concentration of fisetin; it was found that fisetin is effective at a concentration of 1 μmol/L or higher. This amount of fisetin can be readily obtained through the daily diet.[34,35] Therefore, we sought to identify the active site of fisetin to guide effective use of the compound. We found that the hydroxyl group at C-3 is crucial for preventing vascular abnormal contraction by fisetin. Based on this result, we consider the hydroxyl group at C-3 of fisetin to be a model for designing the active site of drugs to preventing and/or treat vascular abnormal contraction.

Therefore, we need to consider plant variety and the place of production to utilize mulberry leaves as an effective food for fisetin intake. Mulberry leaves may be an excellent source of compounds with health benefits for vascular tissue, and thus, contribute to the extension of life span and reduce medical costs. However, further studies are needed to investigate the biological activities of fisetin, evaluate its short- and long-term safety and effectiveness in animal models and analyze its effects in humans through clinical studies.

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CONFLICT OF INTEREST
The authors declare no potential conflict of interest.

AUTHOR CONTRIBUTIONS
Natsuko Tsurudome performed the main experiments and analysis and drafted the manuscript. Yuji Minami reviewed and supervised the study. Katsuko Kajiya identified the compound by instrumental analysis, designed

the research, raised funding, supervised the project, and edited the paper. All authors have read and approved the final manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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